PCT WORLD INTELLECTUAL P Internatio

WO 9602665A1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/04, 1/06, 1/48, 1/66

A1

(11) International Publication Number: WO 96/02665

(43) International Publication Date: 1 February 1996 (01.02.96)

(21) International Application Number: PCT/GB94/01513

(22) International Filing Date: 13 July 1994 (13.07.94)

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Whitehall, London SW1A 2HB (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: SQUIRRELL, David, James [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(74) Agent: BREAKELL, Amy; DRA, R69 Building, Farnborough, Hampshire GU14 6TD (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

Published

With international search report.

With amended claims.

(54) Title: MICROBIOLOGICAL TEST METHOD AND REAGENTS

(57) Abstract

A method for determining the presence and/or amount of microorganisms and/or their intracellular material present in a sample comprising estimating the amount of adenylate kinase therein by its ability to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP) in the presence of added magnesium ions and relating that to the presence/or amount or organism and/or intracellular material. The method provides improved sensitivity over existing luciferase/luciferin assays. Reagents including purified ADP and adenylate kinase free luciferase are provided together with test kits including these and apparatus for automated operation of the method.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

MICROBIOLOGICAL TEST METHOD AND REAGENTS

The present invention relates to a method for detecting and assaying microorganisms, to agents for use in such a method, and to test kits comprising essential reagents for carrying out the method.

All living organisms utilise adenosine triphosphate (ATP) as a source of chemical energy and it is known to assay this using the ATP driven luciferase/luciferin reaction. Light generated by this enzymic reaction can be measured using a luminometer and related to the amount of ATP present. The usefulness of ATP as an index of microbial numbers has been known since the mid 1960's (see ATP Luminescence Rapid Methods in Microbiology (1989) editor Stanley et al.; Blackwell Scientific Publications, London, see pages 1-10); its main advantage being speed and sensitivity. Utilising this assay format simple samples can be analysed in a matter of minutes while complex ones routinely take only half an hour with a detection capability provided down to 10^{-12} mol/l ATP. There is however a need for methods which provide still further sensitivity when detecting microorganisms or their contents while retaining speed and ease of performance.

The present inventor has now determined that the speed and sensitivity of ATP based method can be enhanced significantly by shifting the target of the assay from ATP to an enzyme which generates it, particularly to adenylate kinase. Adenylate kinase is an enzyme used by all organisms for the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The targeting of this enzyme in preference to ATP, by using the preferred method, reagents and kits of the invention, allows the detection of down to at least 10^{-20} moles of intracellular marker adenylate kinase.

It is known to assay adenylate kinase using the luciferase/luciferin system (see Brolin et al Journal of Biochemical and Biophysical Methods 1 (1979) 163-169 and Shutenko et al. Biotekhnologiya, No 4,

(1988) 542-547) for the purpose of determining its activity and this has been applied to study of certain mammalian and plant tissues (eg. see Rodionova et al Fiziologiya Rastenii (1978) 25, 4, P731-734). The use of such assay system for the detection and assay of microorganisms however has not been suggested and the advantages of doing such, ie. enhanced sensitivity so provided, have not been relevant to those studying the enzyme itself.

Although adenylate kinase is present in smaller quantities than ADP or ATP, its use as a biological marker for microorganisms provides enhanced sensitivity with a typical amplification available of 400,000 by measuring its presence through the ATP it produces; that is for every mole of enzyme present 400,000 moles of ADP are converted to ATP in a 10 minute incubation. Thus estimation of the enzyme by measuring the substrate or product of the reaction it catalyses provides for detection down to as low as 10^{-20} moles.

The applicant's copending PCT application PCT/GB94/00118 relates to the general method of estimation of microorganisms in a sample from the sample's ability to convert ADP to ATP, and relating that to the presence of microorganisms or their intracellular materials. That application exemplifies the method wherein magnesium ions, necessary for the reaction of two molecules of ADP with each adenylate kinase active site, are not added as a reagent but are provided by any bacteria cells present and as an impurity in the other reagents. The number of cells detected in the examples using such technique proved to be about 10^2, with results of a more statistically valid nature being obtained at 10^3 or more; a linear relationship between luminometry counts and cell numbers then being obtainable.

The present invention relates to an improved technique in which adenylate kinase activity is measured in an optimised fashion using reaction conditions in which magnesium ions are supplied to the ADP conversion reaction, and wherein the reagents used are treated to

remove adenylate kinase to higher degrees of purity whereby the number of microorganisms that can be detected is of the order of tens rather than hundreds per 200µl sample, and readings with linear relation between cells and ATP derived light are possible down to 10 cells.

In a first aspect of the present invention there is provided a method for determining the presence and/or amount of microorganisms and/or their intracellular material present in a sample characterised in that the amount of adenylate kinase in the sample is estimated by mixing it with adenosine diphosphate (ADP), determining the amount of adenosine triphosphate (ATP) produced by the sample from this ADP, and relating the amount of ATP so produced to the presence/or amount of adenylate kinase and to microorganisms and/or their intracellular material, wherein the conversion of ADP to ATP is carried out in the presence of magnesium ions at a molar concentration sufficient to allow maximal conversion of ADP to ATP. The amount of magnesium present is preferably such that there is sufficient to provide one mole of magnesium for one mole of ADP such that all of the ADP molecules may be associated with at least one magnesium ion.

In preferred embodiments of this aspect of the invention the sample is provided in the form of an aqueous suspension or solution and the estimation of adenylate kinase, and thus microorganisms and/or intracellular material in the sample, is carried out by adding ADP and magnesium ions to the sample under conditions whereby any adenylate kinase present will convert ADP to ATP, incubating the sample for a predetermined period to effect such conversion, adding luciferase and luciferin agents, determining the amount of light emitted from the sample and relating that to presence and amount of adenylate kinase.

The amount of ADP with which the sample is mixed is preferably sufficient to provide an ADP concentration in the mixture in excess of 0.005mM, more preferably in excess of 0.01mM and most preferably in excess of 0.08mM. A particularly preferred amount of ADP in the

conversion step mixture is about 0.1mM.

Where reagents are to be used which contain magnesium ion depleting agents, eg. chelating/sequestering agents such as EDTA and phosphate buffers, it will be realised that in order to provide the ADP with sufficient magnesium ions for it to undergo optimal conversion, it will be preferred for an excess of magnesium ions to be present. For the preferred concentrations of ADP set out above, the preferred concentration of magnesium ions in the suspension or solution during conversion of ADP to ATP is 1mM or more, more preferably 5mM or more and most preferably 10mM or more. The magnesium ions may be provided in the form of any magnesium salt, preferably as magnesium acetate.

A further preferred format of the present invention adds luciferin /luciferase luminometry reagents to the sample at the beginning of the incubation, preferably as a single reagent with the ADP and magnesium ion source. Lucifease is preferably stored separately from extractant.

In formats of the invention where all the reagents are included at the start of the conversion of ADP to ATP in this manner, and/or where luminometer counting is continued after luciferin/luciferase addition where that is a separate step, magnesium may be provided by the luciferin/luciferase reagent. However, due to binding of magnesium ions by EDTA and phosphate it is necessary that the amount of magnesium ions is positively ensured by prior experiment or calculation. It will be realised by those skilled in the art that the optimal amount of magnesium salt to be added to a given ADP, sample and luciferin /luciferase mixture will be readily determinable by routine experiment using a sample containing a known amount of bacteria, eg. E. coli. whereby maximal signals are obtained. Figure 3 below gives an indication of the optimal amount of magnesium acetate to be added to a mixture as used in the Examples below.

As Mg^{2+} ions facilitate ADP depletion due to contaminant adenylate

kinase, it is preferred not to keep them in solution together prior to use; chelating agent such as EDTA may be included in the ADP to prevent this. Preferably the magnesium and ADP are brought together just prior to use or in the ADP conversion step. Where the reagents are to be kept together it is preferred that they are kept in freeze dried form to avoid any premature ADP conversion to ATP.

As stated above, although other assays may be used, ATP is preferably detected by use of the luciferin/luciferase system to provide a photometrically detectable signal indicative of the amount of ATP in the sample. Luciferin/luciferase preparations and methods for their use in assaying ATP will be well known to those skilled in the art and are commercially available (eg. see Brolin et al). A typical formulation contains eg. 0.1 to 10mg/litre luciferase, 15 to 1000µmol/litre D-luciferin, and agents such as $MgCl_2$ (2.5-25mmole), EDTA, BSA, and pH7 buffer (see eg. EP 054676).

For single reagent use with adenylate kinase testing methods as described herein it is preferred that the pH is adjusted to that which is optimal for both enzymes, ie. a compromise, in order that counting might continue while converting ADP to ATP. This may be determined by routine experiment using known bacterial numbers in a sample.

The sample, ADP and magnesium ion source may be mixed in any buffer providing a pH suitable for the adenylate kinase reaction; no other reagents are necessary. Thus any buffer providing a pH of between 5.5 and 8.5 might be used, with optimal pH lying between pH6 and 7, preferably pH6.5. Examples of suitable buffers include Tris and phosphate buffers. Most suitably the sample is collected and/or diluted in such a buffer in preparation for carrying out the method of the invention.

As with any amplified assay, the sensitivity of the adenylate kinase assay of the present invention is limited by the purity of the

reagents. In this case the significant contaminants are ATP in the ADP substrate and adenylate kinase in the luciferase preparation. For use as a sensitive assay for microorganisms, particularly where these may be potentially harmful and need detecting in low numbers, it is necessary that the purity of each of the reagents be as high as possible with respect to the substance with which it is to react in the assay.

To address the first problem, high purity commercial ADP (>99.5% purity) is preferably used after further purification by column chromatography. This is desirable because even small amounts of contaminating ATP may be sufficient to cause a high background reading. For example, using a diethylaminoethylcellulose column and 0.02mM hydrochloric acid eluent, ATP is eluted more slowly from the column than ADP to a degree enabling substantial separation. Other chromatographic media and eluent combinations may also be used to similar effect, for example HPLC using Nucleosil column packings (available from Technicol, Stockport Cheshire UK), such as Nucleosil 3 and Nucleosil 5, using 0.06M KH_2PO_4:methanol in 77:23 ratio v/v at pH6 with 5mM tetrabutylammonium hydrogen sulphate. Fractions with high ADP to ATP ratios are retained for use and purity is assessed by luciferin/luciferase reagent mediated bioluminescence after adenylate kinase action to measure ADP levels and without adenylate kinase to measure ATP contaminant levels.

Using a preferred Econopaq Q strong anion exchange gel cartridge (Biorad) equilibrated with 20mM potassium phosphate at pH4.6 and eluting with steps of KP_i concentration up to 400mM, ADP was found to be strongly retained and eluted as a coherent peak, with ATP eluting after it. In this manner ADP with a molar % ATP upper limit of 2×10^{-8} was obtainable. The most pure ADP the applicants are aware of from the literature is 0.001% (see Shutenko et al, as above) thus the present invention provides ADP for use in the method of the present invention that has less than 0.001 molar % ATP, more preferably 2×10^{-8} molar % or less.

A further method for removing ATP from the ADP substrate uses enzymes that specifically degrade ATP, such as luciferase or apyrase. Such enzymes may also be used to further purify chromatographically purified ADP, or alternatively enzymically purified ADP may be treated by column chromatography. It will be noted that apyrase is also an ADPase, but as some are more active on ATP and the ADP is present at much higher levels this does not present a significant problem.

With regard to the second problem, adenylate kinase, as an essential "housekeeping" enzyme, is present in virtually all organisms and is generally present in luciferase preparations. It may only be a minor contaminant, but since the aim is to measure very low adenylate kinase levels in samples, its presence in the luciferase may be a limiting factor. In fact the applicant has determined that, defining one unit (U) of activity as the amount of enzyme which converts 1µmol ADP to 1µmol ATP per minute in the presence of 0.5mM ADP and 4.5mM Mg^{2+} at pH7.8 at 20°C, commercial luciferases may contain 10^{-7}U/ml or more adenylate kinase activity whereas luciferin, its substrate, comprises very little if any activity. Furthermore it is common to stabilise luciferase reagents with a stabiliser, commonly a protein such as bovine serum albumin (BSA), and commercial preparations of this have been determined by the applicant to possess significant adenylate kinase activity.

The molecular weights of luciferase and adenylate kinase are significantly different, being 61kD and 21kD respectively. Furthermore luciferase is a membrane bound protein and therefore relatively hydrophobic, whereas adenylate kinase occurs as a soluble enzyme. It is thus possible to remove adenylate kinase from luciferase preparations by, eg. size exclusion chromatography, reverse phase chromatography, or both. Alternatively or in addition to this, the problem of adenylate kinase contamination of luciferase can be avoided by adding the bioluminescent reagents (luciferase and luciferin) just before or as measurements are taken so that any contaminating

adenylate kinase does not have the time to produce a significant effect.

Suitable methods for purifying luciferase use column chromatography fractionation with a low porosity gel, eg Sephadex G-25 (see Nielsen and Rasmussen, Acta Chemica Scandinavica 22 (1968) p1757-1762; use of Sephadex and Sepharose columns (eg Blue Sepharose) in series and/or SDS electrophoresis (see Devine et al, Biochimica et Biophysica Acta 1172 (1993) 121-132) or ageing for a period at elevated ambient temperature.

In order to remove adenylate kinase activity from agents such as bovine serum albumin it is similarly possible to use column chromatography. A further treatment that has proved successful in this regard is chemical treatment of the BSA such that its ability to stabilise the luciferase is retained, but adenylate kinase activity is reduced or depleted altogether. Any conventional chemical treatment for the depletion of enzymic activity from proteins may be equally be applied for this purpose. Alternatively a non-protein luciferase stabiliser, eg. glycerol, may be used as a supplement or replacement for the BSA.

For example, the applicant has determined that commercially available BSA can have its adenylate kinase activity reduced to less than 2% of its original activity or less merely by heat treatment at acid or alkaline pH. One suitably effective treatment heats the BSA at pH5.6 or pH10 at 50°C for 24 hours. A further source of adenylate kinase free BSA is the chemically treated reagent acetylated-BSA, available from Sigma and BDH. It will be realised by those skilled in the art that other chemically treated BSAs will also be suitable.

In order to render all the adenylate kinase associated with a target microorganism available to the ADP, magnesium ions and luciferase /luciferin assay reagents of the invention it will be necessary to disrupt them such that intracellular material is released or otherwise exposed to the reagents. Such disruption might be carried out using

mechanical means such as an ultrasonic generator, by use of osmotic shock optionally in association with cold shock or such agents as lysozyme or, more conveniently, by use of detergents. Such detergents are commercially available and commonly referred to as 'extractants'. Typical extractants include generic cationic detergents such as CTAB (cetyl trimethyl ammonium bromide), and proprietory agents such as Biotrace XM extractant (available from Biotrace, Bridgend UK), Celcis UK cationic extractants and Lumac NRM (nucleotide releasing agent available from Lumac BV, Holland). When using CTAB a convenient preparation will include 0.01 to 1% CTAB in water. eg 0.2%, but other concentrations may occur to those skilled in the art.

Thus before adding ADP and luciferase/luciferin reagent(s) to an assay sample suspected of containing microorganisms it is preferred to disrupt these to render their intracellular contents accessible to luminometry reagents by use of disrupting agent. If it is desired to distinguish between target cells and cells such as those of fungal spores it is possible to run two separate assays treating one with a nonionic detergent capable of disrupting only these spores and multi -cellular 'somatic' animal cells (eg.Triton TX-100) and the other with cationic detergent 'extractants' detailed above for disrupting all cells. It is possible to carry out these assays on the same sample if an ATPase such as apyrase is added between detergent/luciferase /measurement cycles; one cycle using nonionic and the other cationic detergent in a first cycle step with filtration steps between.

The effect of extractant upon the luciferase/luciferin system is known to be important (see eg. Simpson et al (1991) J. Biolumin Chemilumin 6(2) pp97-106); with cationic detergents being known to potentiate the reaction but to cause gradual inactivation of luciferase, anionic detergent inhibiting the reaction and nonionic and zwitterionic detergents being known to potentiate over a wide range. A mixture of 0.15% cationic detergent together with 0.25% tertiary diamine surfactant (obtained from Celcis, Cambridge, UK) was found to be

satisfactory for present purposes, but those skilled in the art will have no problem screening for other 'extractants' that yield an optimal mix of adenylate kinase and luciferase activity when copresent in the same solution.

The light given off from the mixture after all the essential steps are complete, ie. ADP conversion to ATP and subsequent action of luciferase upon luciferin, may be measured by residence of the sample volume, eg. luminometer tube, within a light detector immediately after or simultaneously with addition of the luciferase and luciferin or other agents which enable the essential steps to proceed.

In a second aspect of the present invention there is provided a test kit comprising the essential reagents required for the method of the invention, ie. adenosine diphosphate, a source of magnesium ions and preferably luciferase and luciferin. Preferably the kit includes all these reagents, with the luciferase and luciferin being provided as a single reagent solution, with a detergent reagent in the kit suitable for disrupting the target cells for which the assay is intended. Usually for assaying microorganisms only cationic detergent is needed, whereas if fungal spores and somatic cells are likely to be significant then a further nonionic detergent reagent might be included to assess their numbers. The kit is in the form of a single package preferably including instructions as to how to perform the method of the invention; the reagents being provided in containers and being of strength suitable for direct use or after dilution.

It may be appropriate to provide the magnesium ions with the luciferase/luciferin reagent if this is to be added before the ADP to ATP coversion has begun, but then they should be in excess over that bound to the EDTA or phosphate in that reagent and should be optimised to accomodate both adenylate kinase and luciferase requirements. For microbial detection magnesium ions are preferably provided with a sample collection/dilution buffer but other formats may be preferred

for particular applications. Most conveniently the magnesium ions are provided with a sample collection or dilution buffer, the ADP is provided with detergent/surfactant extractants and optionally with a stabiliser such as EDTA, and the luciferase and luciferin are provided together, thus providing a three reagent test kit. Alternatively these agents may be provided in the form of a single reagent that is freeze dried such that they do not interact to cause degredation of eg. the ADP, prior to use.

A preferred test kit of the invention comprises ADP reagent which is of purity higher than 99.999%, and a luciferase/luciferin reagent, including BSA, that is substantially free of adenylate kinase activity. Alternatively the luciferase/luciferin ratio used, reflected in the kit instructions for use and/or in their relative concentrations, is such that the luciferase is capable of acting upon the luciferin substrate sufficiently quickly such that any luciferase associated adenylate kinase produces ATP after the initial emission is finished; thus microorganism derived adenylate kinase will be indicated by a flash kinetic reaction and contaminant ATP by a glow.

The preferred purified reagents may be provided by the methods described above. It is noted that adenylate kinase activity in luciferase may also be depleted by leaving the luciferase to stand for a period of months or years.

The methods, apparatus, reagents and kits of the present invention will now be illustrated by way of example only with reference to the following non-limiting Examples and Figures. Further embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

Figure 1: plots log luminometer signal against log number of E. coli

in a 200 µl sample using the improved assay of the invention using 1 and 5 minute incubations prior to luciferin/luciferase addition.

Figure 2: plots log luminometer signal against log number of cells of E. coli in absence of magnesium.

Figure 3: shows the effect of magnesium ion concentration upon the luminometer signal derived from a set number of P. aeruginosa at pH 7.5 and pH 8.0 showing the 10 fold increase over no added magnesium.

Example 1: Preparation of purified adenosine diphosphate reagent.
Liquid chromatography was used to further purify commercial high purity (>99.95%) ADP (Sigma) using a 5ml Econopac Q cartridge (Biorad) equilibrated with 20mM potassium phosphate pH4.6 and loaded with 5ml of the 1mM ADP (2.1 mg). Elution was carried out by steps of KP_i concentration up to 400mM whereupon the ADP was strongly retained and eluted as a peak at approximately 340mM KP_i. Setting the system up with a pump (5ml/min) and gradient mixer, a gradient of 50 to 1M KP_i in 200ml total was provided and 5ml fractions collected. ADP eluted as a sharp peak between fractions 12 and 17 with ATP beginning to appear at the end of the gradient. A step in [KP_i] to 1 M eluted the remaining ATP. The purest ADP fractions from this column were of less than $2x\ 10^{-8}$ mole % ATP.

Example 2: Preparation of adenylate kinase free luciferase reagents.
Adenylate kinase activity was deleted from commercially available luciferin/luciferase reagents (Biotrace HM) by ageing, including several months at high ambient temperature (circa 30°C) over a period of 12 months in dry form.

Example 3: Alternative preparation of kinase free luciferase reagents.
Commercially available luciferase is purified using column chromatography by the method of Devine et al (1993) using Blue Sepharose as referred to above.

Example 4: Preparation of adenylate kinase free BSA.

Sigma Fraction V (RIA Grade, Cat. No. A-7888) BSA was made up at 1% weight/volume in 200ml sterile water to give a starting pH of 5.6. Two 50ml samples of this were put into 100ml Duran bottles, the remainder made to pH10 using 5M NaOH and 50ml put into each of two Durans. Thimerosal was added to 0.02% final concentration as a preservative to prevent microbial growth and the bottles incubated at 37°C or 50°C for 24 hours before the pH of each was readjusted to 7.6 with 5M HCl or 5M NaOH as appropriate. Adenylate kinase activity was measured by mixing 100µl BSA sample as prepared above with 100µl 30mM magnesium acetate solution, placing the resultant mixture in a 3.5ml luminometer tube in a luminometer and adding 100µl ADP solution prepared in Example 1 and 100µl luciferin /luciferase reagent (Celcis, Cambridge UK) that was prepared free of adenylate kinase activity by use of column chromatography and chemically treated BSA. After a 5 second delay light emission integrated over 10 seconds was measured and recorded on a computer and a total of 10 sequential 10 second readings were made to determine the rate of ATP production; analyses being performed in duplicate. Calibration was made using 4 replicate measurements of the light emitted from 5µl of 10ng/ml (91 femtomoles) of ATP in water: mean signal 2950 per femtomole.

Results: The BSA sample incubated at 37°C remained clear whilst those at 50°C formed a precipitate which was slight at pH10 and very heavy at pH5.6. At pH10 and 50°C there was slight discolouration. Adenylate kinase activity remaining in these samples is shown below in Table 1 below as represented by luminometer counts per minute.

It is recommended that still milder forms of inactivation are used, with longer duration, or that the BSA is immediately freeze dried, if it is intended to store it for any length of time as after 2 weeks even the pH10 50°C sample became unuseable due to increased discolouration. Th 37°C samples did not go off in this manner and thus offer better scope for reducing stable adenylate kinase free BSA

by increasing the incubation time. The fact that the Biotrace HM agent lost its activity in dry form after storage at 40°C demonstrates the possibilities here.

TABLE 1: Treatment	Counts t5-15	t95-105	Difference	d[ATP]/dt ($fm.sec^{-1}$)
37/5.6	9350	41727	23207	7.1
	9845	26041	(means)	
	11896	32945		
37/10	7192	30602	17943	5.5
	5047	20557		
	4469	19377		
50/5.6	606	1191	595	0.18
	343	948		
50/10	460	1014	500	0.15
	342	847		
	314	754		

Example 5: Preparation of luciferin/luciferase reagent with BSA.

Commercial preparations of luciferin/luciferase commonly contain BSA as necessary. BSA chemically treated as set out in Example 4 above or as commercially available as acetylated BSA (eg BDH or Sigma) was admixed with adenylate kinase free luciferase in normal proportions together with other standard Celcis agents such as to provide a Celcis LDR luciferin/luciferase luminescence reagent of adenylate kinase activity less than 10^{-9}U assay volume (ie. 300µl).

Example 6: Test kit of the invention.

A test kit of the invention is provided consisting of the following:

(i) a container holding 15mM magnesium acetate solution for collection /dilution of samples;

(ii) a container holding purified ADP solution (>99.99999998% pure with regard to ATP) prepared as described in Example 1 in a concentration of 0.3mM in potassium phosphate (7.5mM pH6.5) buffer solution further including 0.2mM EDTA and a mixed extractant of 0.15% cationic detergent and 0.25% tertiary diamine surfactant.

(iii) a container holding luciferin/luciferase LDR (Celcis, Cambridge, UK) bioluminescence reagent of adenylate kinase activity less than 10^{-8}U/100µl.

Optionally included in the package is a container of nonionic detergent solution (Triton X-100 0.2% or equivalent) and/or a container holding an ATPase such as apyrase for the destruction of ATP released by the action of the nonionic detergent on a sample rendering it suitable for reassay by addition of the cationic detergent.

Example 7: Assay of known amounts of E.coli using method of the invention.

A one week old E. coli broth culture containing approximately 2.2 x 10^7 microorganisms per 200µl of phosphate buffered saline pH7.4 was used as stock and diluted in successive dilutions of 10 with the collection/dilution reagent containing magnesium ions ((i) in Example 6) to give a range of samples of from 10^7 to 0.1 microorganisms per 200µl sample.

Each 200µl sample was added to a 3.5ml luminometer tube, 100µl of ADP/extractant reagent ((ii) in Example 6) added and the mixture, total volume 300µl, was incubated at room temperature for 1 or 5 minutes. On completion of the incubation 100µl of modified Celcis LDR bioluminescence reagent, ((iii in Example 6 above) was added and the light emitted determined over a first 10 second interval and then over 10 second intervals up to one minute to determine the increase in light in cumulative fashion using a Biotrace M3 luminometer. The initial signal value was subtracted from the final reading to gain a measure of the signal in counts per minute.

The efficacy of the present method can be seen by reference to Figure 1 where statistically valid linear response between number of E.coli and light emitted by the sample mix after 5 minute incubation with ADP is obtained for 10 organisms per sample and upward, for 100 organisms and upward with a 1 minute incubation, and a detection limit of about 10 organisms is given in both cases. This compares very favourably with the method of PCT/GB94/00118 which gives a difference of only 26 cpm after a 1 minute incubation with 100 organisms and 67 cpm with 1000; linear responses only being obtained with 1000 cells and over. By comparison 1000 cells per sample in the present method gives a signal increase of several thousand cpm after 1 minute.

It will be realised that in order to perform an assay for an unknown number of microorganisms in a sample using the present method, a calibration curve may be provided plotting known numbers of microorganisms against luminometer counts as shown in the Figures 1 and 2 (eg. as log values), deriving a number of counts from a sample containing the unknown number of microorganisms (including zero organisms) using the same protocol, and estimating the number of microorganisms in the sample as being that corresponding to the same number of counts on the curve.

It will be realised by those skilled in the art that the amount of adenylate kinase present in a particular microorganisms, eg.bacteria, may vary from other microorganisms. For example, yeasts contain more adenylate kinase than bacteria by virtue of their size, and indeed single yeasts can be detected by this method. Thus for a given microorganism a particular calibration curve may be required, and it may be necessary to provide such curves for different states of the same microorganism, eg. for weakened, pH or oxygen stressed organisms. However, a further advantage of the present method over exitsing ATP based methodology is that adenylate kinase content will be more closely correlated to cell numbers than the highly variable ATP content which is depleted by cell metabolism.

CLAIMS.

1. A method for determining the presence and/or amount of microorganisms and/or their intracellular material present in a sample characterised in that the amount of adenylate kinase in the sample is estimated by mixing the sample with adenosine diphosphate (ADP), determining the amount of adenosine triphosphate (ATP) produced by the sample from this ADP, and relating the amount of ATP so produced to the presence/or amount of microorganisms and/or their intracellular material, wherein the conversion of ADP to ATP is carried out in the presence of magnesium ions at a molar concentration sufficient to allow maximal conversion of ADP to ATP.

2. A method as claimed in claim 1 wherein the amount of magnesium ions present is such that there is sufficient to provide one mole of magnesium for one mole of ADP such that all of the ADP molecules may be associated with at least one magnesium ion.

3. A method as claimed in claim 1 or 2 wherein the sample is provided in the form of an aqueous suspension or solution and the estimation of microorganisms and/or intracellular material therein is carried out by adding ADP and magnesium ions to the sample under conditions whereby any adenylate kinase present will convert ADP to ATP, incubating the sample for a predetermined period to effect such conversion, adding luciferase and luciferin agents, determining the amount of light emitted from the sample and relating that to presence and amount of microorganisms and/or intracellular material.

4. A method as claimed in any one of claims 1 to 3 wherein the amount of ADP with which the sample is mixed is sufficient to provide an ADP concentration in the mixture in excess of 0.005mM.

5. A method as claimed in claim 4 wherein the ADP is in excess of 0.08mM.

6. A method as claimed in claim 4 wherein the ADP concentration is about 0.1mM.

7. A method as claimed in any one of the preceding claims wherein the concentration of magnesium ions in the suspension or solution during conversion of ADP to ATP is 1mM or more.

8. A method as claimed in claim 7 wherein the concentration of magnesium ions in the suspension or solution is 10mM or more.

9. A method as claimed in any one of the preceding claims wherein the magnesium ions are provided in the form of magnesium acetate.

10. A method as claimed in any one of the preceding claims wherein the luciferin/luciferase luminescence reagents are added to the sample at the beginning of the incubation as a single reagent with the ADP and magnesium ion source.

11. A method as claimed in any one of the preceding claims wherein the magnesium ion source and the ADP are kept in dry form or in separate solutions prior to use and brought together or made into an aqueous solution immediately prior to use or in the ADP conversion step.

12. A method as claimed in any one of the preceding claims wherein the magnesium ion source and sample are mixed together before adding the ADP.

13. A method as claimed in claim 12 wherein the sample is collected or diluted in a solution comprising the magnesium ion source.

14. A method as claimed in any one of the preceding claims wherein the conversion of ADP to ATP is carried out at a pH of between 5.5 and 8.5.

15. A method as claimed in any one of the preceding claims wherein the ADP has a molar % ATP of less than 0.001%.

16. A method as claimed in claim 15 wherein the ADP has a molar % ATP of 2×10^{-8} or less.

17. A method as claimed in claim 15 wherein the ADP is stored in the presence of a chelating agent to prevent contaminant adenylate kinase converting it to ATP prematurely.

18. A method as claimed in any one of the preceding claims wherein the luciferase/luciferin reagent has an adenylate kinase content of less than 10^{-7}U/ml.

19. A method as claimed in claim 18 wherein the luciferase/luciferin reagent comprises bovine serum albumin that has been chemically treated to deplete its adenylate kinase activity.

20. A method as claimed in any one of the preceding claims wherein the sample is treated with an extractant which disrupts microorganism cells and exposes their adenylate kinase to the ADP and magnesium ions.

21. A method as claimed in claim 20 wherein the cells are fungal spores or somatic cells and the extractant comprises a non-ionic detergent.

22. A method as claimed in claim 20 wherein all cells are to be detected and/or quantified and the extractant comprises a cationic detergent.

23. A method as claimed in claim 22 wherein the extractant further comprises a surfactant.

24. A method as claimed in claim 20 wherein the cells are bacterial cells wherein the ATP released by non-ionic detergent is subtracted from the ATP released by cationic detergent and surfactant, and the remainder related to bacterial cell numbers.

25. A test kit for detection and/or quantification of microorganisms and/or the cellular material comprising ADP and a source of magnesium ions together with an extractant for exposing microorganism adenylate kinase to these such that conversion of the ADP to ATP takes place.

26. A test kit as claimed in claim 25 further comprising luciferase and luciferin in the form of a bioluminescence reagent capable of emitting light in the presence of ATP.

27. A test kit as claimed in claim 26 wherein the source of magnesium ions is provided as a sample collection or dilution solution.

28. A test kit as claimed in claim 27 wherein the collection or dilution buffer comprises magnesium acetate.

29. A test kit as claimed in any one of claims 26 to 28 wherein the ADP is together with detergent and/or surfactant extractant.

30. A test kit as claimed in any one of claims 26 to 29 wherein the ADP, the magnesium ion source and the bioluminescence agent are provided in three separate containers.

31. A test kit as claimed in any one of claims 26 to 29 wherein the agents are all provided as a single freeze dried reagent.

32. A test kit as claimed in any one of claims 25 to 31 wherein the ADP is of purity higher than 99.999 mole % with respect to ATP.

33. A test kit as claimed in any one of claims 25 to 32 comprising a bioluminescence reagent of adenylate kinase activity less than 10^{-7}U/ml.

34. A test kit as claimed in claim 32 wherein the bioluminescence reagent comprises bovine serum albumin that has been chemically treated to deplete its adenylate kinase activity.

35. A reagent comprising ADP of purity with respect to ATP of greater than 99.999%.

36. A reagent as claimed in claim 35 further comprising a chelating agent in sufficient amount to prevent contaminating adenylate kinase from converting ADP to ATP.

37. A reagent as claimed in claim 36 wherein the chelating agent comprises EDTA.

AMENDED CLAIMS

[received by the International Bureau on 29 September 1995 (29.09.95); original claims 1-37 replaced by amended claims 1-45 (6 pages)]

1. A method for determining the presence and/or amount of micro-organisms and/or their intracellular material present in a sample characterised in that the amount of adenylate kinase in the sample is estimated by mixing the sample with adenosine diphosphate (ADP), determining the amount of adenosine triphosphate (ATP) produced by the sample from this ADP, and relating the amount of ATP so produced to the presence/or amount of micro-organisms and/or their intracellular material.

2. A method as claimed in claim 1 wherein the amount of ADP with which the sample is mixed is sufficient to provide an ADP concentration in the mixture in excess of 0.005mM.

3. A method as claimed in claim 2 wherein the ADP is in excess of 0.08mM.

4. A method as claimed in claim 3 wherein the ADP concentration is about 0.1mM.

5. A method as claimed in any one of claim 1 to 4 wherein the conversion of ADP to ATP is carried out in the presence of magnesium ions at a molar concentration sufficient to allow maximal conversion of ADP to ATP.

6. A method as claimed in claim 5 wherein the amount of magnesium ions present is such that there is sufficient to provide one mole of magnesium for one mole of ADP such that all of the ADP molecules may be associated with at least one magnesium ion.

7. A method as claimed in claim 5 or 6 wherein the sample is provided in the form of an aqueous suspension of solution and the estimation of micro-organisms and/or intracellular material therein is carried out by adding ADP and magnesium ions to the sample under conditions whereby any adenylate kinase present will convert ADP to ATP, incubating the sample for a predetermined period to effect such conversion, adding luciferase and luciferin

agents, determining the amount of light emitted from the sample and relating that to presence and amount of micro-organisms and/or intracellular material.

18. A method as claimed in any of claims 5 to 7 wherein the concentration of magnesium ions in the suspension or solution during conversion of ADP to ATP is lmM or more.

9. A method as claimed in claim 8 wherein the concentration of magnesium ions in the suspension or solution is 10mM or more.

10. A method as claimed in one of claims 5 to 9 wherein the magnesium ions are provided in the form of magnesium acetate.

11. A method as claimed in any one of claims 5 to 10 wherein the luciferin/luciferase luminescence reagents are added to the sample at the beginning of the incubation as a single reagent with the ADP and magnesium ion source.

12. A method as claimed in any one of claims 5 to 11 wherein the magnesium ion source and the ADP are kept in dry form or in separate solutions prior to use and brought together or made into an aqueous solution immediately prior to use or in the ADP conversion step.

13. A method as claimed in any one of claims 5 to 12 wherein the magnesium ion source and sample are mixed together before adding the ADP.

14. A method as claimed in claim 13 wherein the sample is collected or diluted in a solution comprising the magnesium ion source.

15. A method as claimed in any previous claims wherein the conversion of ADP to ATP is carried out at a pH of between 5.5 and 8.5.

16. A method as claimed in any previous claims wherein the ADP has a molar % ATP of less than 0.001%.

17. A method as claimed in claim 17 wherein the ADP has a molar % ATP of 2×10^{-8} or less.

18. A method as claimed in any previous claim wherein the ADP is stored prior to use in the presence of a chelating agent to prevent contaminant adenylate kinase converting it to ATP prematurely.

19. A method as claimed in claim 7 or any claim dependant thereon wherein the luciferase/luciferin reagent has an adenylate kinase content of less than 10^{-7}U/ml.

20. A method as claimed in claim 19 wherein the luciferase/luciferin reagent comprises bovine serum albumin that has been chemically treated to deplete its adenylate kinase activity.

21. A method as claimed in any previous claim wherein the sample is treated with an extractant which disrupts micro-organism cells and exposes their adenylate kinase to the ADP and magnesium ions.

22. A method as claimed in claim 21 wherein the cells are fungal spores or eucaryotic cells and the extractant comprises a non-ionic detergent.

23. A method as claimed in claim 21 wherein all cells are to be detected and/or quantified and the extractant comprises a cationic detergent.

24. A method as claimed in claim 21, 22 or 23 wherein the extractant further comprises a surfactant.

25. A method as claimed in claim 21, 22, 23 or 24 wherein the cells are bacterial cells wherein the ATP released by non-ionic detergent is subtracted from the ATP released by cationic detergent and surfactant, and the remainder related to bacterial cell numbers.

26. A test kit for detection and/or quantification of micro-organisms and/or the cellular material comprising one or more reagents including a reagent comprising ADP with a molar % ATP of less than 0.001.

27. A test kit as claimed in any of claim 26 further comprising luciferase and luciferin n the form of a bioluminescence reagent capable of emitting light in the pesence of ATP.

28. A test kit as claimed in claim 26 or 27 further comprising a source of magnesium ions.

29. A test kit as claimed in claim 28 wherein the source of magnesium ions is provided as a sample collection of dilution buffer solution.

30. A test kit as claimed in claim 29 wherein the collection or dilution buffer solution comprises magnesium acetate.

31. A test kit as claimed in any of claim 26 to 30 further comprising an extractant suitable for acting upon micro-organisms such as to expose micro-organism adenylate kinase to ADP in solution therewith such that conversion of the ADP to ATP takes place.

32. A test kit as claimed in any of claims 26 to 31 wherein the ADP is in a reagent together with a detergent and/or surfactant extractant.

33. A test kit as claimed in any of claim 26 to 32 wherein the ADP, a magnesium ion source and a bioluminescence reagent are provided in three separate containers.

34. A test kit as claimed in any of claims 26 to 33 wherein the reagents are all provided as single freeze dried reagents.

35. A test kit as claimed in any of claims 26 to 34 wherein the ADP is of purity of 99.99999998% or more with respect to ATP.

36. A test kit as claimed in 27 or 33 comprising a bioluminescence reagent of adenylate kinase activity less than 10^{-7}U/ml as defined herein.

37. A test kit as claimed in claim 27 or 33 wherein the bioluminescence reagent comprises bovine serum albumin that has been chemically treated to deplete its adenylate kinase activity.

38. A test kit as claimed in sny of claims 26 to 37 wherein the ADP reagent further comprises a chelating agent in sufficient amount to prevent contaminating adenylate kinase from converting ADP to ATP.

39. An apparatus comprising a means for receiving a sample to be analysed for the presence of micro-organisms or their intracellular contents in an aqueous suspension or solution, means for addition of ADP, luciferase and luciferin to the suspension and means for detecting light produced wherein a conveyor is provided for moving the sample and means relative to each other for the purpose of sequential operation.

40. An apparatus as claimed in claim 39 further comprising a means for adding a source of magnesium ions to the sample prior to the means for detecting light produced.

41. An apparatus as claimed in claim 39 or 40 further including a means for adding detergent to the suspension before a means for adding the luciferase and luciferin.

42. An apparatus as claimed in claim 41 wherein the ADP reagent is added with the detergent.

43. An apparatus as claimed in any one of claim 39 to 42 further including a light detecting station where luciferase and luciferin are added to the sample prior to monitoring light emitted therefrom with the means for detecting light.

44. An apparatus as claimed in any one of claims 39 to 42 comprising a conveyor means which receives a volume of liquid medium holding the sample and carries it through one or more reagent stations to the light detection means.

45. An apparatus as claimed in any one of claim 39 to 42 comprising a conveyor adapted to receive a series of luminometry vessels which are preloaded with an aqueous liquid suspension of material to be tested for the presence of micro-organisms or which are passed through a station of the apparatus where such suspension is placed therein.

Fig.1.

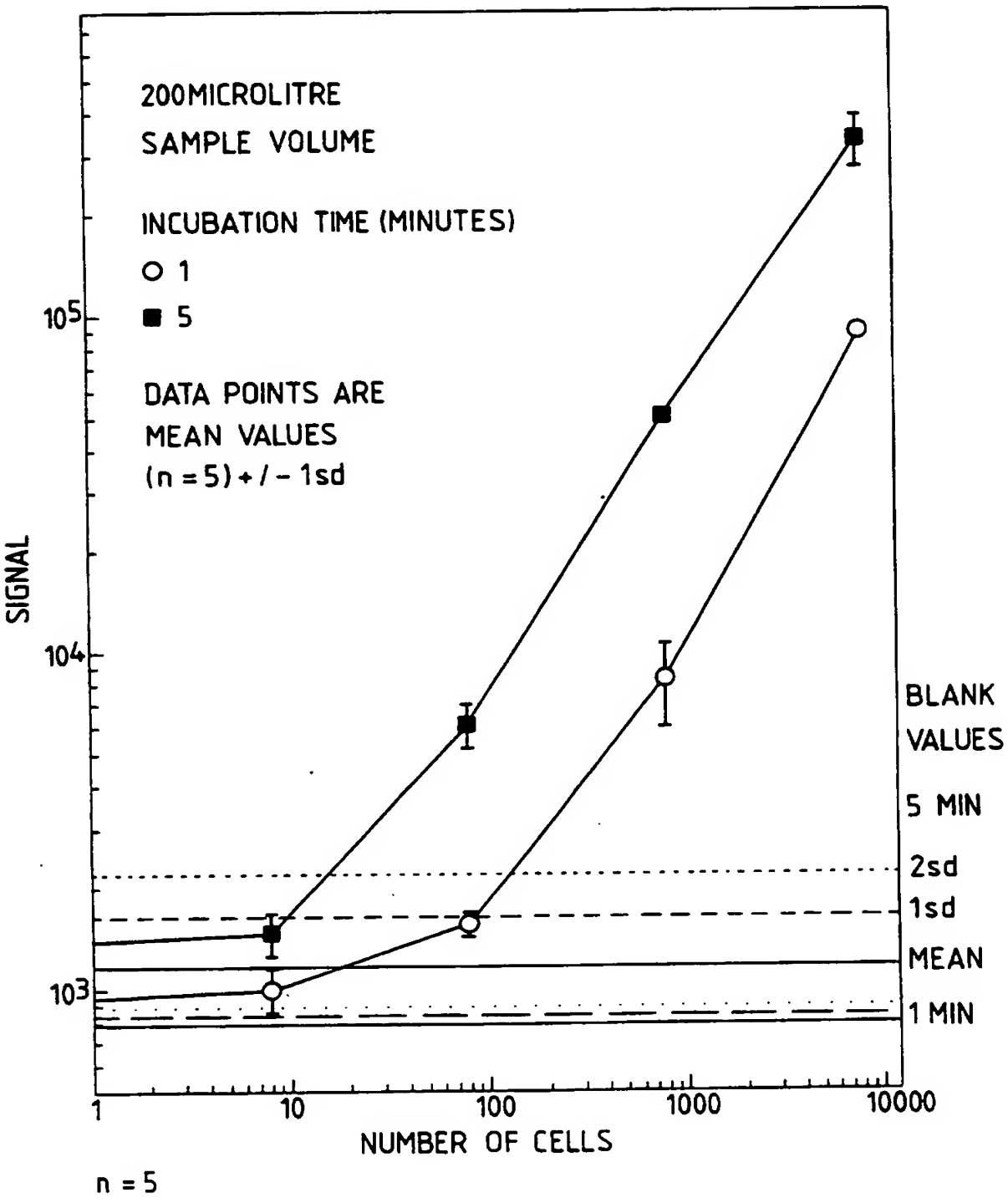


Fig.2.

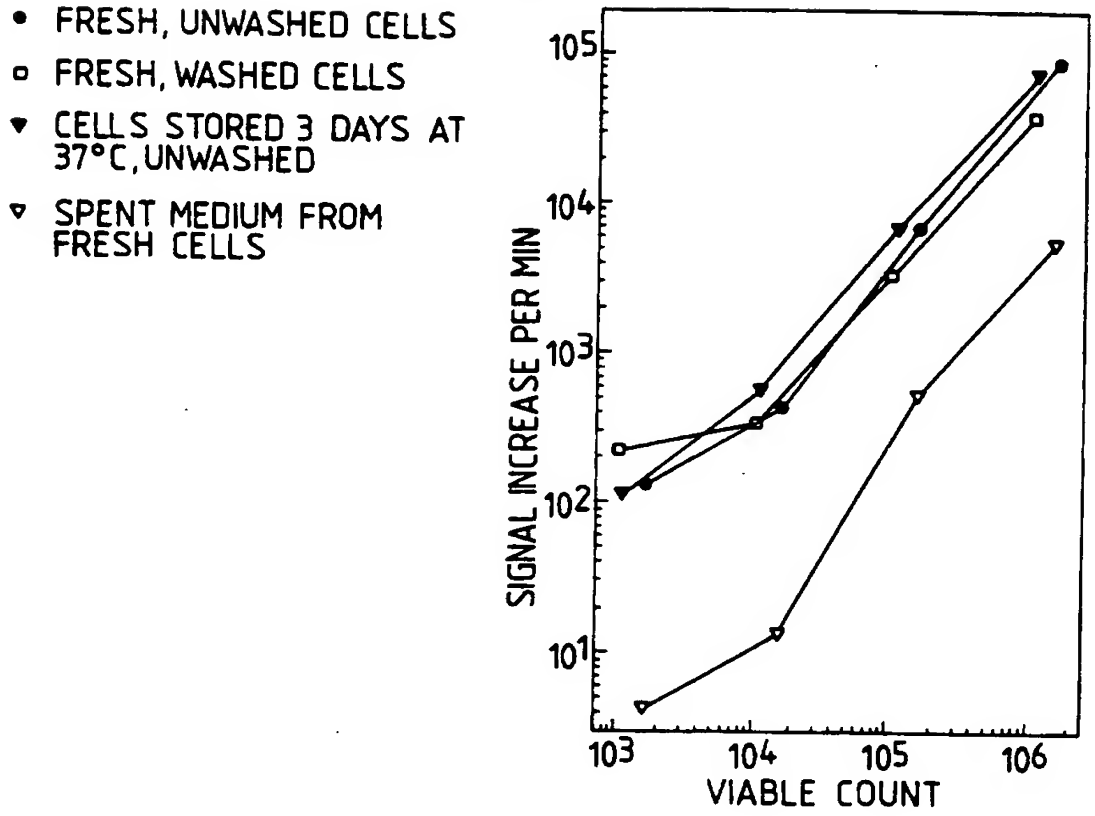
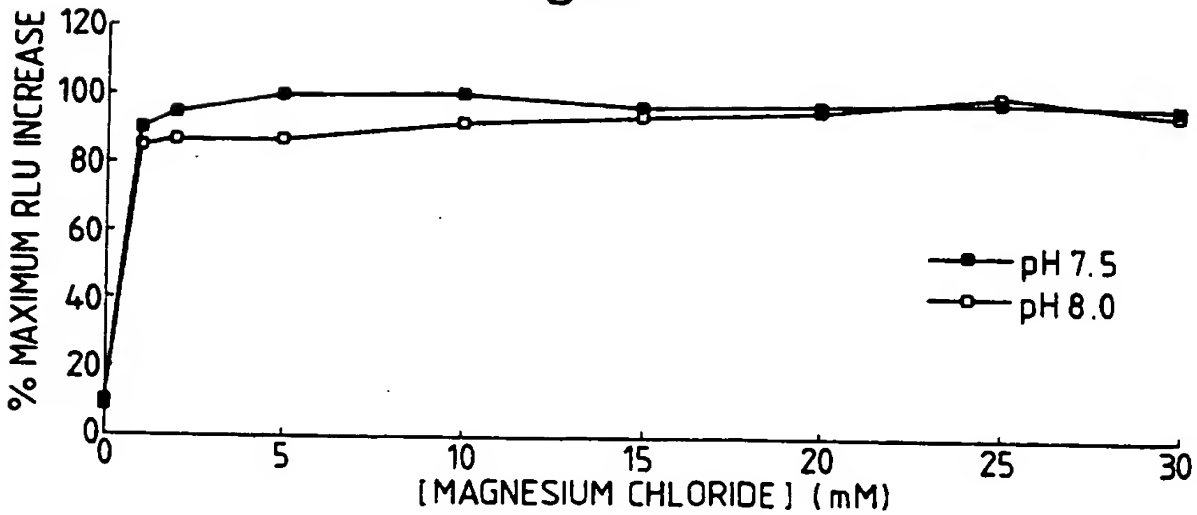


Fig.3.



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/01513

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/04 C12Q1/06 C12Q1/48 C12Q1/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,94 17202 (THE SECRETARY OF STATE FOR DEFENCE IN THE GOVERNMENT OF THE UK) 4 August 1994 cited in the application see the whole document ---	1,3-6, 9-37
A	H.U. BERGMEYER 'Methods of Enzymatic Analysis, Volume III, Third Edition' 1986 , VERLAG CHEMIE , WEINHEIM, DE see Chapter 7.5.3 :"Adenylate Kinase - Luminometric Method" by Sven E. Brolin see page 553 - page 559 --- -/--	1-19, 25-37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 1995	29. 03. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Döpfer, K-P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01513

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, vol.1, no.3, 1979, AMSTERDAM, NL pages 163 - 169 SVEN E. BROLIN ET AL. 'Photokinetic microassay of adenylate kinase using the firefly luciferase reaction' cited in the application see the whole document ---	1-20, 25-37
A	CH,A,678 065 (HAMILTON BONADUZ AG) 31 July 1991 see the whole document ---	1,3,10, 18-26, 30,31
A	EP,A,0 054 676 (BOEHRINGER MANNHEIM GMBH) 30 June 1982 cited in the application see example 1 ---	1,3,18, 19,34, 36,37
A	US,A,3 933 592 (JOHN C. CLENDENNING) 20 January 1976 see the whole document -----	1,3,18, 20-26

INTERNATIONAL SEARCH REPORT

In' tional Application No
PCT/GB 94/01513

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9417202	04-08-94	AU-B-	5841794	15-08-94
CH-A-678065	31-07-91	NONE		
EP-A-0054676	30-06-82	DE-A-	3047860	15-07-82
		AT-T-	5201	15-11-83
		JP-C-	1422202	29-01-88
		JP-A-	57131063	13-08-82
		JP-B-	62032421	14-07-87
US-A-3933592	20-01-76	NONE		